THE RELATIONSHIP OF ANTIGENIC COMPONENTS IN EGG-JELLIES OF VARIOUS AMPHIBIAN SPECIES [1, 2]

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In recent years, interactions between specific complementary surface substances, which are presumed to act in antigen-antibody-like fashion, have been postulated to account for a number of different kinds of specific intercellular reactions. Spiegel's investigation (1955) on the inhibition of reaggregation of dissociated embryonic cells and of dissociated sponge cells, tissue-incompatibility studies (Woerdeman, 1955), and tissue-affinity studies (Townes and Holtfreter, 1955) are examples of the type of cellular interaction which might be interpreted on the basis of complementary surface configurations.

Perhaps the most thoroughly investigated cells with reference to specific interacting substances are the spermatozoa and eggs of various marine invertebrates, particularly echinoderms. Studies of these substances have been given considerable attention in the past few years (for recent reviews, see Tyler, 1959; Metz, 1957, 1961; Runnström *et al.*, 1959). Evidence from several lines of investigation indicates that "fertilizin," the substance of the sea urchin egg-jelly, performs a significant if not essential role in fertilization (Tyler, 1955; Metz, 1957, 1961).

Likewise, several lines of study suggest that the jelly material surrounding amphibian eggs performs an essential function in fertilization. Thus, amphibian eggs without jelly, either as they normally occur in the body cavity or after removal of the jelly artificially, are not fertilizable (Bataillon, 1919; Kambara, 1953; Tchou-Su and Wang, 1956; Shaver and Barch, 1960; Subtelny and Bradt, 1961). Since jellyless body cavity eggs of the frog are capable of normal cleavage after artificial activation, either by inoculation of a cellular element (Bataillon, 1919), or after transfer of a blastula nucleus (Subtelny and Bradt, 1961), it is clear that the egg-jelly layer is not essential for development. It is reasonable to assume, then, that the jelly layer is involved in one or more essential interactions with the sperm in the process of normal fertilization. This assumption is supported by the observation that jellyless eggs can become fertilizable when artificially enrobed by jelly capsules taken from ovulated eggs (Subtelny and Bradt, 1961). Finally, Shaver and Barch (1960) have shown that antiserum prepared against the jelly-coat material of *Rana pipiens* will inhibit fertilizability of eggs of the same species. Since the jelly-coat material is immunologically tissue-specific (Shaver, Barch and Shivers, 1962) it appears that such inhibition of fertilization results from action of antiserum upon the egg-jelly material. In studies using nonprecipitating, univalent anti-egg-jelly sera, Shivers and Metz (1962) again obtained inhibition of fertilizing

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capacity in frog eggs. These results with univalent antibodies indicated an actual blocking of egg-jelly receptor sites that perform some essential interaction with the sperm at fertilization. In view of this evident importance of the amphibian egg-jelly in fertilization, a more detailed description of this material seemed warranted. The present report shows that several antigenic components are present in amphibian egg-jellies. Some of these are common to several species, whereas others are restricted to a few or even a single species.

Materials and Methods

Jelly-coat material was mechanically removed with watchmaker forceps, subsequent to hydration in distilled water, from mature unfertilized eggs of four species of frogs (*R. pipiens, R. clamitans, R. sylvatica* and *R. catesbeiana*). The jelly was washed several times with distilled water and lyophilized until dry with a Virtis freeze-mobile. Standard antigen solutions for injection into rabbits were prepared by blending ten mg. of this lyophilate with one ml. of 0.85% sodium chloride, buffered at pH 7.4 with Sorenson's phosphate mixture, to which sodium ethyl mercurithio-salicylate (merthiolate, Lilly) was added in a proportion of one part per 10,000. Antigen solutions were also prepared, in the same manner, from egg-jelly capsules of species of Anura (*Bufo americanus* and *Bufo marinus*), as well as from another order of Amphibia (*Ambystoma maculatum*). An antigen preparation of fertilizin obtained by acid extraction (Tyler, 1956) from eggs of *Arbacia punctulata* (Echinodermata), which was kindly supplied by Dr. C. B. Metz, was also available.

Blood for control serum was drawn from the marginal ear vein of large albino rabbits (2.7 kilograms average weight) prior to the injection of antigen. No cross-reaction has been observed between normal rabbit serum and any of the amphibian egg-jelly solutions thus far tested. One and one half ml. of the standard antigen solution emulsified with an equal volume of Freund's complete adjuvant were injected *via* the subscapular route for the production of antisera. A booster injection consisting of the same amount of antigen and adjuvant was repeated 10 days later. A trial bleeding for the presence of antibodies was made three weeks after the second injection. If antibodies appeared at this time, bleedings from the ear vein were continued every other week for 6 to 8 weeks. Generally 3–5 rabbits were given injections with the same preparation and sera from these rabbits were pooled.

The antigenic components of the various jellies were analyzed by the agar-gel diffusion technique (Ouchterlony, 1949), slightly modified (Shaver, 1961). After agar plates were prepared in the usual way, various arrangements of wells were made, into each of which 0.75 ml. of the reactants was placed. The plates were developed for 5 days at room temperature (20–22° C.) and photographed for a permanent record.

In order to remove or neutralize specific antibodies the antisera were absorbed by mixing them with various dilutions of inhibiting antigen in glass tubes for 24 hours at 4° C. The antigen-antiserum mixture was centrifuged at 10,000 *g* to remove any precipitate which formed. The supernatant was then used as a test antiserum. Preliminary tests showed that an equal volume of the standard antigen preparation was sufficient to render serum non-precipitating. Unabsorbed

antibody preparations were mixed with an equal volume of Sorenson's phosphate mixture before analyzing to maintain equal dilutions in the absorbed and unabsorbed antisera.

Results

Analysis of antigens in species of Rana: Agar-gel diffusion precipitin analysis of the antigenic components of egg-jellies of the four *Rana* species revealed similar patterns to the extent that the jelly of each species contained at least five distinct antigens. One of these antigens was common to all four species. Each of the species had a unique combination of cross-reacting antigens and two or three species-specific antigens. The relationships for anti-jelly serum of *R. pipiens* are illustrated in Figure 1. This figure is a drawing of a fully-developed agar diffu-

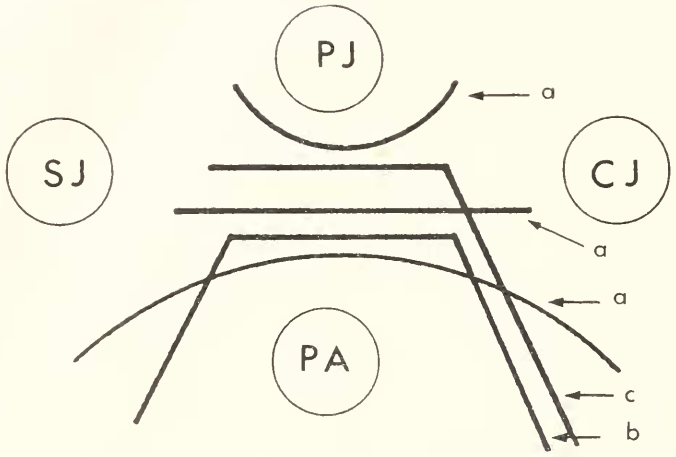

Figure 1. Diagram of double diffusion plate, showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens* (PA) with the egg-jelly material of *R. sylvatica* (SJ), *R. pipiens* (PJ) and *R. clamitans* (CJ). a, b and c = precipitation bands representing components which are species-specific, shared among the three species and shared between only two of the species, respectively.

sion plate in which unabsorbed anti-jelly serum of *R. pipiens* (Well PA) was reacted with antigens prepared from egg-jelly material of *R. sylvatica* (Well SJ), *R. pipiens* (Well PJ) and *R. clamitans* (Well CJ). Those components which are present in the jelly of eggs of *R. pipiens* are represented by lines formed between the antiserum well and well PJ (*e.g.,* lines a, b and c, Figure 1). The three lines labelled a are species-specific to *R. pipiens.* The curvature of the line nearest the antiserum well does not represent a cross-reaction between the antiserum and the heterologous jellies. The component common to the egg-jellies of all three species is indicated by the continuous line extending between the antiserum well and the three antigen wells (line b, Figure 1). Components present in the egg-jellies of *R. pipiens* and *R. clamitans,* but not present in the jelly of eggs of *R. sylvatica,* are represented by a continuous line between the antiserum well and wells CJ and PJ (*e.g.,* line c, Figure 1), but not present opposite well SJ.

These relationships of jelly antigens were confirmed by appropriate absorption

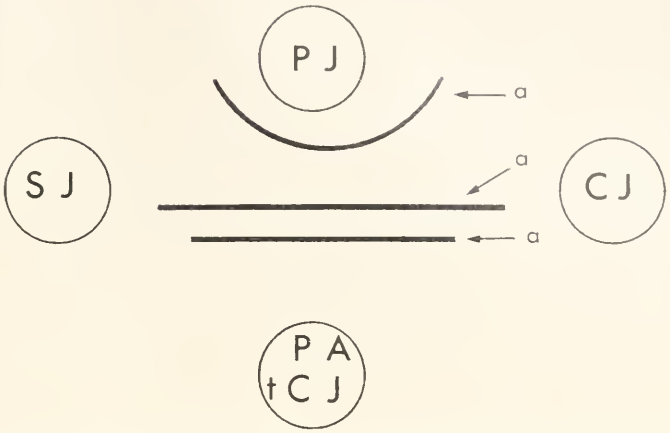

FIGURE 2. Diagram of double diffusion plate showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens,* which had previously been absorbed with jelly of *R. clamitans* (PA+CJ), with jelly antigens shown in Figure 1. a = precipitation bands representing components which are species-specific.

of anti-jelly sera prior to the testing of these sera on agar-gel plates. As expected, antiserum failed to produce any precipitin lines following absorption with egg-jelly of the homologous species. In addition, antiserum prepared against the egg-jelly of one species was absorbed with jelly material from eggs of other species. Such absorbed serum was then diffused against egg-jelly solutions of several species (Figs. 2 and 3). Absence of precipitin lines between the absorbing heterologous jelly and the absorbed serum (*e.g.,* wells CJ and PA + CJ, Figure 2; wells SJ and PA + SJ, Figure 3) served as a check for complete absorption. The spectrum of lines between the homologous jelly and the absorbed serum represented those antigens not present in the absorbing heterologous jelly. Some of these repre-

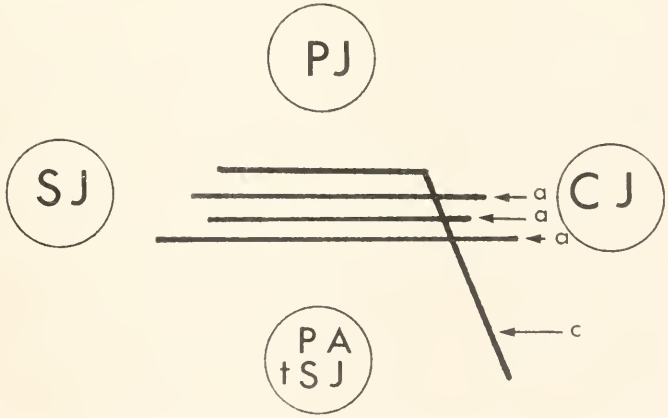

FIGURE 3. Diagram of double diffusion plate showing precipitation bands formed by reacting anti-jelly serum of *R. pipiens,* which had previously been absorbed with jelly of *R. sylvatica* (PA+SJ), with jelly antigens shown in Figure 1. a and c = precipitation bands representing components which are species-specific and shared between two of the species, respectively.

sented species-specific antigens since the lines appeared even after absorption and failed to join with lines of the other heterologous species (*e.g.*, lines a, Figures 2 and 3). Finally, joining of precipitin lines of the homologous and additional heterologous jellies indicated sharing of antigens not present in the original absorbing heterologous species (*e.g.*, line c, Figure 3).

By means of similar analysis employing antisera made against the egg-jellies of *R. clamitans, R. sylvatica* and *R. catesbeiana,* the relationships of the antigenic components in the egg-jellies of these species were ascertained. The results are

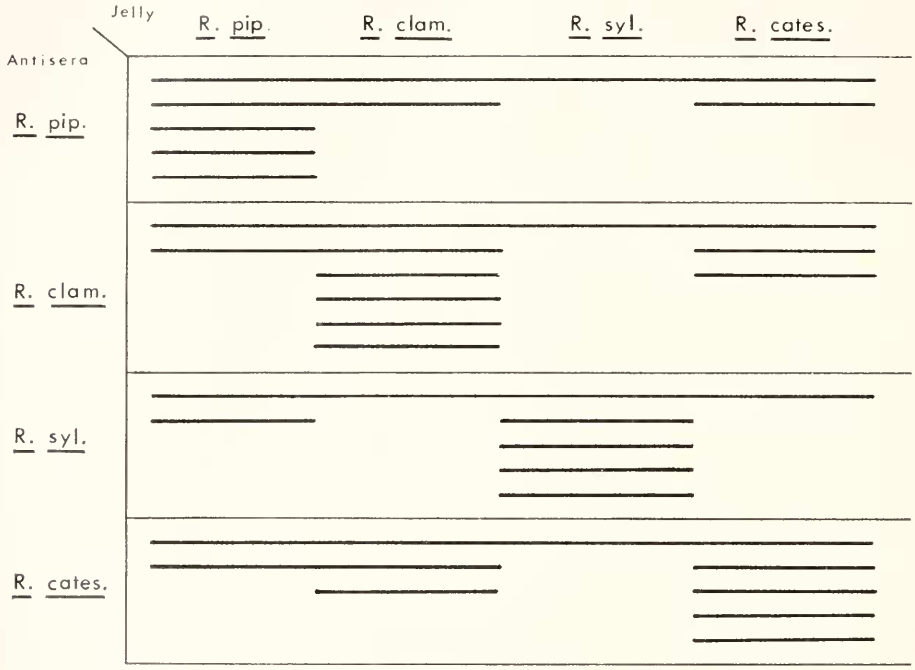

FIGURE 4. Interrelationships of antigens in egg-jellies of species of *Rana* as determined by agar-gel diffusion analysis. Antigens were determined by reacting the anti-jelly serum of each species with the various jellies. Common antigens are represented by continuous lines. Lines for each species represent a minimum number of antigenic components.

diagrammed in Figure 4. The figure shows a component shared among all four species of *Rana*; a component shared among *R. pipiens* and *R. clamitans* and *R. catesbeiana* which is not present in the jelly of *R. sylvatica;* a component shared between *R. clamitans* and *R. catesbeiana* which is not present in jellies of *R. pipiens* or *R. sylvatica;* and a number of components which are species-specific in each case. A component common to *R. pipiens* and *R. sylvatica* could be detected by the anti-jelly serum of *R. sylvatica* but not by the *R. pipiens* antiserum.

In addition to the interrelationships among the egg-jelly antigens of the four species of *Rana,* jellies of some more distantly related species were examined. These were *Bufo americanus, B. marinus, Ambystoma maculatum* and the echinoderm, *Arbacia punctulata.* These results are presented in Table I.

A number of antigenic components were observed which were specific to the jelly of *B. americanus,* as well as other antigens which were shared between this species and *B. marinus.* Cross-reactions were also observed between antiserum against *B. americanus* egg-jelly and the jellies from the four species of *Rana.* When reciprocal combinations of the jelly antigens of *B. americanus* and the anti-jelly serum of *A. maculatum, R. clamitans* and *R. catesbeiana* were made to react, no precipitin bands appeared.

Diffusion of *Bufo marinus* jelly against homologous antiserum resulted in at least five precipitin bands. Some represented specific antigens, whereas others represented antigens shared with *B. americanus.* In contrast to *B. americanus,* when *B. marinus* egg-jelly antiserum was reacted with jelly antigens from the species of *Rana,* no cross-reactions were observed.

TABLE 1

Antigenic components in egg-jellies of species of Rana and Bufo (Anura), Ambystoma (Urodela) and Arbacia (Echinodermata; Echinoidea) as determined by agar-gel diffusion precipitin tests

Anti-egg-jelly serum of \ Jelly	*B. amer.*	*B. mar.*	*A. mac.*	*Arb. punc.*	*R. syl.*	*R. cates.*	*R. pip.*	*R. clam.*
Bufo americanus	5	3	2[a]	0	1	2[a]	2	2[a]
Bufo marinus	3	5	0	0	0	0	0	0
Ambystoma maculatum	0[b]	0	5	0	0[b]	2[a]	0	0
Arbacia punctulata	0	0	0	2	0	0	0	0

Numbers represent precipitin bands which appeared by reacting the anti-jelly serum with the jelly. a. reciprocal reaction produced no bands. b. reciprocal reaction produced two precipitin bands.

The antiserum prepared against the egg capsules of *Ambystoma maculatum* produced five precipitin bands when made to react with the homologous jelly antigen. When the antiserum of this urodele species was reacted with the jelly antigens of the four species of *Rana,* and of the two species of *Bufo,* only the jelly of *R. catesbeiana* produced bands. The reciprocal combination of jelly antigens of *A. maculatum* with the anti-jelly serum of *B. americanus* and *R. sylvatica* produced two precipitin bands.

The antiserum prepared against a sample of "fertilizin" made from jelly material of eggs of *Arbacia punctulata* produced two specific precipitin bands when reacted with the homologous jelly but gave no visible reactions with jelly antigens from any of the species of Amphibia.

DISCUSSION

The species-specificity of fertilization is well nigh an axiom of biology. This specificity, combined with the high correlation between cross-fertilization and cross-agglutination of spermatozoa by substances from eggs, especially in echinoderms (*cf.* Tyler, 1959, for earlier work on this topic), suggests that there are specific molecular patterns on the surface of gametes which interact during fertilization. In addition to the specificity factor of fertilization, other roles in fertilization have been attributed in part to the interaction of egg and sperm surface

substances. These include attachment of sperm to the egg, initiation of acrosomal reaction, sperm engulfment and activation of the egg. The egg-jelly material of both echinoderms and amphibians has been suggested as one of the complementary egg surface substances which interacts with the sperm at fertilization. Additional evidence for the egg-jelly of amphibians being important in the initial steps in fertilization is the fact that antibodies prepared against these jelly antigens inhibit the fertilizability of eggs (Shaver and Barch, 1960; Shivers and Metz, 1962). These observations suggest that surface components in amphibian gametes may represent the same mechanism for the insurance of specificity of fertilization in this group that the "fertilizin-antifertilizin" system may represent in the echinoderms.

The specificity of fertilization in amphibians is not absolute, but allows a certain degree of cross-fertilization between species (for a summary of the variety of crosses which have been made among amphibians, see Moore, 1955). Crosses between species of *Rana* employed in the present study (*R. pipiens, R. clamitans, R. sylvatica* and *R. catesbeiana*) are capable of some development with the exception of crosses involving the ova of *R. clamitans*. It may be inferred that one of the factors in the successful union of gametes of different species would be the degree of similarity of configurations on the surface of gametes participating in cross-fertilization. Thus, if the jelly-coat material of the amphibian egg plays a role in the specificity of fertilization one would expect to find similar substances in the jelly of eggs of species capable of hybridization. It should be noted that where cross-fertilization has been reported to occur between species of *Rana* whose egg-jelly antigens were analyzed in this study, common antigenic components were found to be present. In addition, common antigenic components were found to be present in the egg-jelly of some species which have not been reported as being capable of cross-fertilization (*e.g.*, an antigenic component shared between the egg-jelly of *R. clamitans* and the heterologous species of *Rana*). It is possible that the sperm of these species are capable of making contact with and penetrating the surface of the ova of *R. clamitans* without the subsequent rotation or further development of the egg.

The reasons for the differences observed between reciprocal reactions (*e.g.*, an antigen common to *R. sylvatica* and *R. pipiens* which could be detected by *R. sylvatica* antiserum but not by *R. pipiens* antiserum) cannot be determined from present experiments.

The species-specificity of fertilization may be of a different order of sensitivity than the identities or similarities of antigenic components as determined by precipitation analysis. The similarity of a jelly antigen between two or more species as determined by immunological procedures may not necessarily imply that cross-fertilization would result from approximating gametes of these species. On the other hand, if these egg-jelly antigens perform a significant role in fertilization, then antibodies against them should combine with and block them at the cell surface, thereby inhibiting fertilization. In other experiments (Shivers, 1961 and unpublished results) it was found by selective absorption of antisera that the fertilizability of eggs of *R. pipiens* was markedly inhibited by treatments with two classes of antibodies: (1) those prepared against the species-specific components of *R. pipiens*; and (2) those prepared against the common component

of all four species of *Rana*. That the species-specific components are the ones most likely to be operative in the initial steps of fertilization is deducible from the fact that the fertilizability of eggs was inhibited most strongly by antibodies against the species-specific components; and these are probably the last jelly components to be laid down on the egg during its sojourn in the oviduct (Barch and Shaver, 1963). As expected, the antibodies against the species-specific components of heterologous egg-jellies (*R. clamitans* and *R. sylvatica*) had no effect on the fertilizability of eggs of *R. pipiens*.

Although much remains to be done to elucidate the role of frog egg-jelly capsules in fertilization, these observations on the interrelationships of antigenic components in the jellies and the effect of antibodies against them on the fertilization reaction offer more evidence for an essential role(s) for the jelly-coat material.

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Summary

1. Antisera were prepared against the jelly-coat material of eggs of several species of *Rana* (*R. pipiens*, *R. clamitans*, *R. sylvatica* and *R. catesbeiana*) and other species of Amphibia (*Bufo americanus*, *Bufo marinus* and *Ambystoma maculatum*). Serological characterizations as to species-specificity of antigenic components found in these jellies have been presented.

2. Analysis showed that the jellies of each species contained a number of species-specific components.

3. Common components were observed in the jelly of species belonging to the same genus (either *Rana* or *Bufo*).

4. In certain cases common components were observed between species of different genera (*Bufo americanus* and each of the species of *Rana*).

5. The results of these studies on the specificity of antigenic components in egg-jellies are discussed in connection with the possible role of these components in the process of normal fertilization.

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